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Proliferation and function of microbodies in the nematophagous fungus *Arthrobotrys oligospora* during growth on oleic acid or D-alanine as the sole carbon source

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Abstract: The nematophagous fungus *Arthrobotrys oligospora* is able to grow on oleic acid or D-alanine as the sole carbon source. During growth on oleic acid, activities of enzymes of the β-oxidation pathway, but not catalase, were induced. In the presence of D-alanine, both D-amino acid oxidase and catalase activities were enhanced. Biochemically and cytochemically, the activities of the above enzymes were assigned to microbodies. The significance of these results in relation to the function of microbodies in trophic hyphae, which are formed during nematode infection, is discussed.

Key words: Arthrobotrys oligospora; Nematophagous fungi; Microbodies; β-Oxidation; Catalase; D-Amino acid oxidase

Introduction

The fungus *Arthrobotrys oligospora* (Fresenius) is capable of capturing nematodes with the aid of specialized three dimensional hyphal networks, known as 'traps' [1]. Adhesion is effected by means of an adhesive layer present on the traps. This is followed by penetration of the cuticle and subsequent invasion of the nematode body by trophic hyphae [2]. During digestion of the nematode contents large lipid droplets accumulate inside the trophic hyphae. This accumulation of lipid is associated with the development of many microbodies. Cytochemically, these microbodies (peroxisomes) have been characterized by the presence of catalase and β-oxidation enzymes [3].

Biochemical studies on the role of microbodies in trophic hyphae are greatly hampered by the difficulty of obtaining sufficient hyphal mass. Therefore, we decided to initiate such studies using normal vegetative hyphae of *A. oligospora*. For this purpose cells were incubated in mineral media supplemented with growth substrates known to induce microbodies in other fungi, namely oleic acid and D-alanine [4]. *A. oligospora*

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appeared to grow well when one or both were present as the sole source of carbon and energy. Growth was associated with proliferation of microbodies inside the cells which appeared to play a key role in the metabolism of both substrates. The results obtained are discussed in relation to earlier observations on nematode/fungus interactions.

Materials and Methods

Growth conditions

A. oligospora Fresenius was maintained on agar surfaces [5]. For shake flask cultures, conidia were collected from agar plates by washing with sterile distilled water and transferred to mineral medium [6] containing 0.25% glucose as a carbon source. Cells from exponentially growing cultures (generally after 24–48 h of incubation) were diluted into fresh media containing either 0.1% oleic acid [7] or 0.5% D,L-alanine as the sole carbon source. The organism was grown in 250 ml or 1 l Erlenmeyer flasks at 28°C on a rotary incubator.

Analytical procedures

Hyphae were collected by filtration and extensively washed in potassium phosphate buffer (20 mM, pH 7.2). Crude extracts were prepared as described before [8]. Activities of catalase [9], cytochrome *c* oxidase [10], isocitrate lyase [11] and D-amino acid oxidase [4] were determined by established methods. AcylCoA oxidase, acylCoA dehydrogenase, 3-hydroxy acylCoA epimerase, 3-oxo-acylCoA thiolase and the multifunctional enzyme (2-enoylCoA hydratase and 3-hydroxy acylCoA dehydrogenase) were determined according to [12]. Cell fractionation was performed according to [10] using 1.2 M sorbitol as an osmotic stabilizer. SDS polyacrylamide gel electrophoresis was performed as described [13]. Protein was determined according to Bradford [14] using bovine serum albumin as a standard.

Electron microscopy

Cells were fixed in 1.5% $KMnO_4$ (w/v) for 20 min at room temperature [15]. Cytochemical detection of catalase and D-amino acid oxidase activities was carried out as described [15]. Immunocytochemistry was performed on ultrathin sections of Lowicryl embedded cells using the protein A/gold method [16]. Polyclonal antibodies raised against thiolase from *Candida tropicalis* and isocitrate lyase and catalase from *Neurospora crassa* which showed cross-reactivity with their *A. oligospora* counterparts, were kindly provided by Prof. W.H. Kunau, University of Bochum, FRG.

Results

Growth

Hyphae of *A. oligospora*, precultured on glucose-containing media, grew well upon their transfer into media containing either oleic acid or D-alanine as the sole carbon source. Light microscopy of cells grown in oleic acid-containing media showed that lipid droplets accumulated inside the hyphae within 2 h. During later stages of cultivation lipid droplets were abundant in cells in the centre of the hyphal floccules, but almost absent or not detectable in hyphal tips. Within 40 h the oleic acid had disappeared from the medium and the cultures entered the stationary phase of growth. A similar pattern was observed for cultures on D-alanine; however, accumulation of lipid droplets was not observed in these hyphae at any stage of the growth.

Biochemistry

Table 1 summarizes the activities of different microbody enzymes detected in crude extracts of either oleic acid, or D-alanine grown cells. As evident from this table, activities of β-oxidation and glyoxylate cycle (as judged by the activity of isocitrate lyase) enzymes were strongly enhanced in oleic acid-grown cells, whereas catalase activity remained very low. As described for other filamentous fungi [12], in *A. oligospora* the first step of fatty acid metabolism was catalyzed by an acylCoA dehydrogenase, instead of an acylCoA oxidase; the oxidase, which is involved in fatty acid oxidation in yeasts, was not detected (data not shown). In hyphae grown on D-alanine-con-

taining media, both catalase and D-amino acid oxidase activities were strongly enhanced (Table 1).

The subcellular localization of the different enzymes involved in oleic acid or D-alanine-metabolism was investigated biochemically by subcellular fractionation methods. After differential centrifugation of homogenates obtained from oleic acid-grown cells, activities of various β-oxidation enzymes and isocitrate lyase were sedimentable and mainly present in the $30000 \times g$ pellet (Table 2). Catalase activities, however, were very low in various fractions. The mitochondrial marker enzyme cytochrome *c* oxidase was mainly present in the $10000 \times g$ pellet (data not shown). Electron microscopy confirmed that mitochondria were predominant in this fraction. The $30000 \times g$ pellet on the other hand mainly contained microbodies (not shown). Fractionation of cells grown on D-alanine-containing medium showed that D-amino acid oxidase activity was mainly present in the $30000 \times g$ fraction (Table 2); catalase, however, was predominantly in the supernatant fraction.

Electron microscopy

Glucose-grown cells of *A. oligospora* contained few small microbodies (Fig. 1a) [6]. After a shift of cells to oleic acid, both the number and size of these organelles rapidly increased concomitant with the development of many lipid droplets inside the cells (Fig. 1b). In cells from the stationary phase of growth, lipid droplets had disappeared again, whereas microbodies remained conspicuous (Fig. 1c). Immunocytochemically, the localization of thiolase (Fig 2a, inset) and isocitrate lyase (not shown) was assigned to these microbody profiles. Catalase could not be detected, either cytochemically (by incubation of cells with DAB/H_2O_2 [15]) or immunocytochemically using the protein A/gold method.

Growth of cells on D-alanine was also associated with proliferation of microbodies (Fig. 1d). Serial sectioning showed that these organelles were often elongated in shape. Cytochemically, the activities of catalase and D-amino acid oxidase were demonstrated in these organelles (Fig. 2a,b). In control experiments performed in the presence of L-alanine reaction products were not observed (Fig. 2b, inset).

Interestingly, during the initial adaptation of the organism to growth on D-alanine numerous hyphal traps developed. In shake flask cultures (200 rpm) they remained present during all stages of cultivation. Trap cells were readily discriminated from normal vegetative cells by their sub-

Table 1

Specific activities of various microbody-borne enzymes in crude extracts of *A. oligospora* grown in batch cultures on mineral media supplemented with different carbon sources

Enzyme	Specific activities in hyphae grown on		
	Glucose/NH_4^+	Oleic acid/NH_4^+	D-Alanine
catalase	9	2	108
D-amino acid oxidase	25	7	99
multifunctional enzyme	20	420	0
epimerase	20	900	0
thiolase	30	480	–
isocitrate lyase	50	160	–

Catalase activity is expressed as ΔE_{240}/min·mg protein, other activities as mU/mg protein; –, not determined.

Table 2

Distribution of different microbody enzymes after differential centrifugation of homogenized hyphae of *A. oligospora* following growth on media containing oleic acid or D-alanine as the sole carbon source

Enzyme	P_3/S_3 ratio following growth on	
	Oleic acid	D-Alanine
catalase	*	0.4
D-amino acid oxidase	–	3.5
multifunctional enzyme	3.2	–
epimerase	3.9	–
thiolase	1.4	–
isocitrate lyase	4.5	–

Catalase activity is expressed as ΔE_{240}/min·mg protein, other activities as mU/mg protein; –, not determined; *, not significant due to very low activities. Hyphae were disrupted with guartz sand, filtered and the homogenates subjected to differential centrifugation. Data are expressed as the ratio of the specific activities present in the $30000 \times g$ supernatant (S_3) and pellet (P_3) fractions.

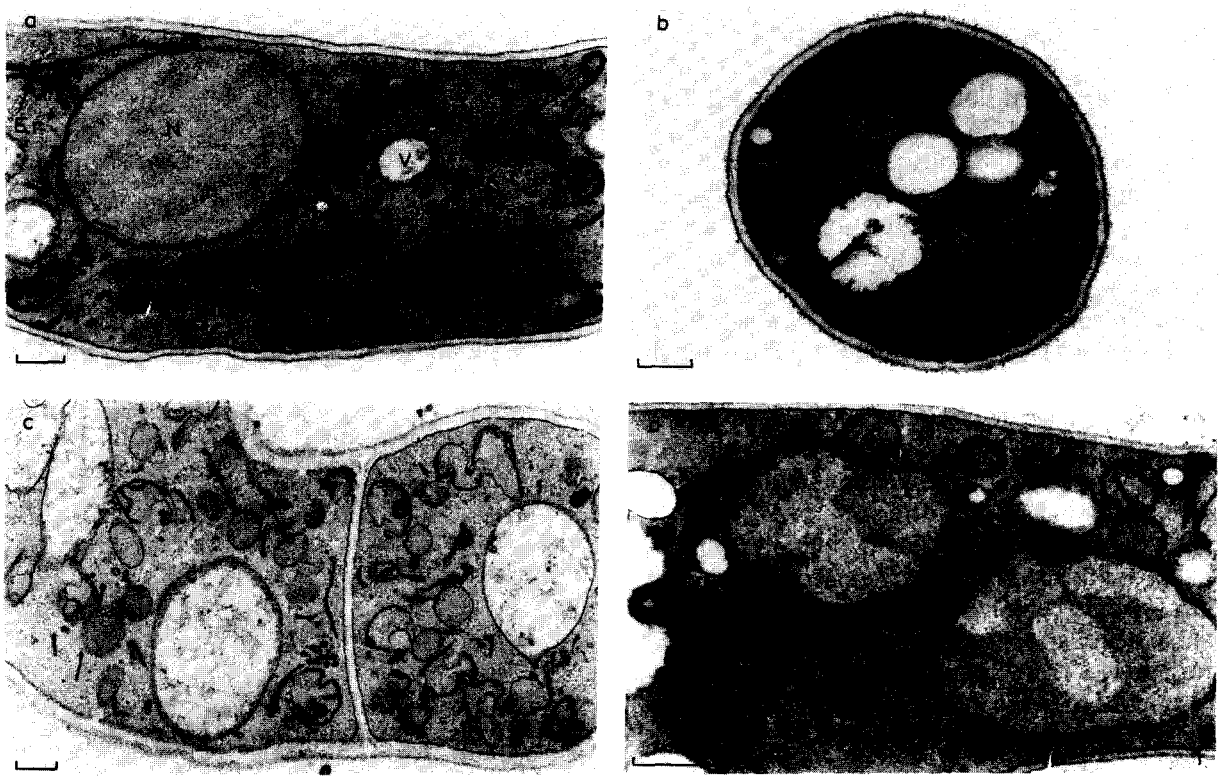

Fig. 1. Survey of cells of *Arthrobotrys oligospora* showing the overall cell morphology during growth on glucose (**a**), oleic acid (**b**) and D-alanine (**d**) as the sole source of carbon. Microbodies (arrows) are small and scarce in glucose-grown cells and abundant in oleic acid- and D-alanine-grown cells. In stationary phase cells, after disappearance of lipid droplets from oleic acid-grown cells, numerous microbody profiles remain detectable (**c**). Abbreviations: M, mitochondrion; N, nucleus; V, vacuole; Er, endoplasmic reticulum. Bar = 0.5 μm.

cellular morphology [6]; they characteristically contained numerous electron dense microbodies (Fig. 2c). The overall structure of the trap cells formed in D-alanine-containing media was fully identical to traps induced by nematodes [6] or small peptides [17]. These observations together with others [18] may suggest that trap formation in *A. oligospora* is induced by adverse conditions.

Discussion

The nematode-trapping fungus *A. oligospora* is able to grow in media containing oleic acid or D-alanine as the sole carbon source. On oleic acid, induction of enzymes of both the β-oxidation pathway and the glyoxylate cycle occurs. These enzymes are located inside microbodies as judged from biochemical and cytochemical criteria. The observation that the first step of fatty acid metabolism is catalyzed by an acylCoA dehydrogenase, instead of an H_2O_2-producing oxidase as in yeasts [12], may offer an explanation for the observed low catalase levels in these cells. The presence of a peroxisomal catalase is not a prerequisite for growth of yeasts on oleic acid. In such cells, H_2O_2 generated in peroxisomes by the acylCoA oxidase, exerts no toxic effects and can be effectively removed by other H_2O_2-degrading pathways [19]. In cells grown on D-alanine, activities of catalase and D-amino acid oxidase are strongly elevated and these enzymes are localized in microbodies. The relatively high soluble catalase fraction observed after cell fractionation most

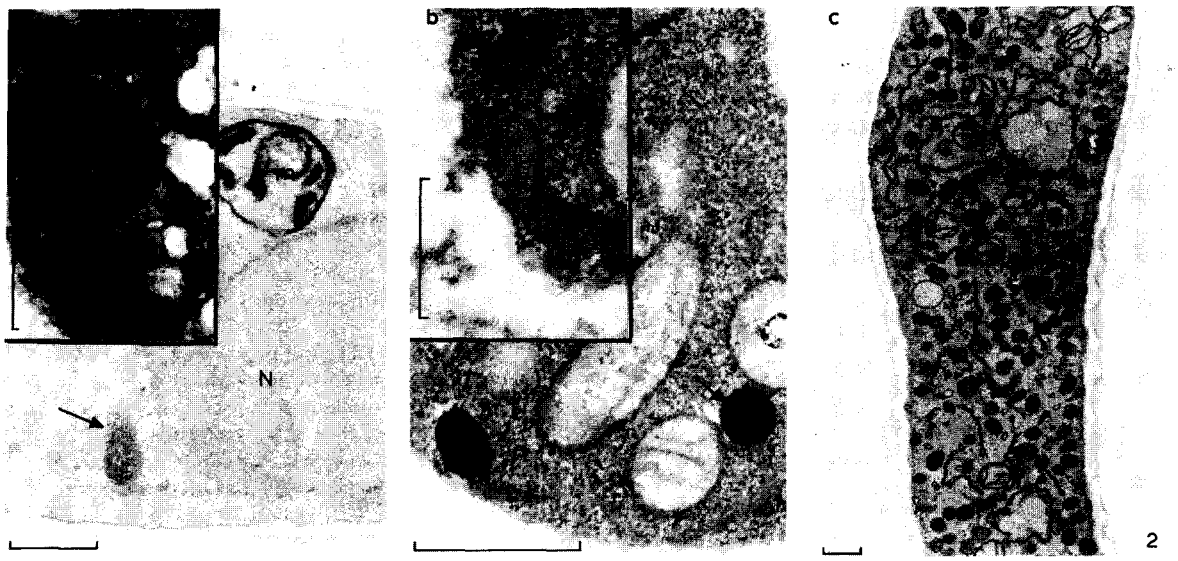

Fig. 2. (Immuno)cytochemistry on oleic acid- and D-alanine-grown cells (**a–c**); labelling of thiolase protein in microbodies in oleic acid-grown cells (**a**, inset); detection of catalase activity (**a**), D-amino acid oxidase activity (**b**) and a control incubated with L-alanine (**b**, inset) in D-alanine-grown cells. Microbodies are indicated by arrows. An overview of a trap cell formed after a shift to D-alanine-containing medium is shown in (**c**); numerous dense bodies are present (arrows). Bar = 0.5 μm.

probably reflects preferential leakage of this enzyme from intact peroxisomes during the isolation procedures [20].

Vegetative hyphae of *A. oligospora* grown on oleic acid are morphologically highly similar to trophic hyphae inside nematodes in that: i) both accumulate lipid droplets concomitant with the proliferation of microbodies and ii) these microbodies contain enzymes of the β-oxidation cycle [3]. However, the presence of catalase activity in microbodies of trophic hyphae suggest that in the latter organelles functions additional to β-oxidation may exist. In fact, microbodies which harbour different metabolic functions simultaneously, have been described before [21]. Therefore, our results are in line with the current view that in fungi the enzymic composition of microbodies reflects environmental conditions.

Apart from 'normal' microbodies, a second class of microbodies, so-called 'dense bodies' is observed in *A. oligospora*. These organelles are confined to trap cells and differ from 'normal' microbodies with respect to their biogenesis, since they are not formed by fission of mature organelles but instead develop from a specialized region of the endoplasmic reticulum [22]. Dense bodies are characterized by the presence of catalase and D-amino acid oxidase activities [6]. These organelles are probably not involved in the primary catabolism of the growth substrate but are shown to play a key role during the initial stages of nematode infection [23]. However, their detailed function is not yet clear. Cloning of genes encoding for β-oxidation enzymes (present in microbodies inside trophic hyphae) or D-amino acid oxidase (which is also present in dense bodies) may provide tools for a molecular approach to study the significance of the different classes of microbodies in *A. oligospora* in depth. Furthermore, peroxisome-deficient (*per*) mutants of yeasts, in which microbodies are completely absent [24], may offer an attractive tool to identify *A. oligospora PER*-genes by functional complementation of these mutants with an *A. oligospora* genomic or cDNA bank. Identification of such genes will allow future studies on the significance of intact microbodies in different cellular functions in nematophagous fungi.

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